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Neutrophil Elastase Promotes Interleukin-1β Secretion from Human Coronary Endothelium*[S]

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Background: The mechanism of IL-1 release from endothelial cells is not fully known.
Results: Neutrophil elastase causes secretion of bioactive IL-1 from endothelial cells via microvesicles.
Conclusion: A mechanistic link between IL-1 secretion from endothelial cells and neutrophil elastase in atherosclerotic plaques is revealed.
Significance: Neutrophil elastase could be a potential target for preventing atherosclerosis.

The endothelium is critically involved in the pathogenesis of atherosclerosis by producing pro-inflammatory mediators, including IL-1β. Coronary arteries from patients with ischemic heart disease express large amounts of IL-1β in the endothelium. However, the mechanism by which endothelial cells (ECs) release IL-1β remains to be elucidated. We investigated neutrophil elastase (NE), a potent serine protease detected in vulnerable areas of human carotid plaques, as a potential "trigger" for IL-1β processing and release. This study tested the hypothesis that NE potentiates the processing and release of IL-1β from human coronary endothelium. We found that NE cleaves the pro-isoform of IL-1β in ECs and causes significant secretion of bioactive IL-1β via extracellular vesicles. This release was attenuated significantly by inhibition of neutrophil elastase but not caspase-1. Transient increases in intracellular Ca^{2+} levels were observed prior to secretion. Inside ECs, and after NE treatment only, IL-1β was detected within LAMP-1-positive multivesicular bodies. The released vesicles contained bioactive IL-1β. *In vivo*, in experimental atherosclerosis, NE was detected in mature atherosclerotic plaques, predominantly in the endothelium, alongside IL-1β. This study reveals a novel mechanistic link between NE expression in atherosclerotic plaques and concomitant pro-inflammatory bioactive IL-1β secretion from ECs. This could reveal additional potential anti-IL-1β therapeutic targets and provide further insights into the inflammatory process by which vascular disease develops.

Atherosclerosis is a complex chronic inflammatory disease that involves inflammatory cell recruitment and release of pro-inflammatory cytokines (1). IL-1β has been implicated in several aspects of vascular inflammation and neointima formation (2). Endothelial cell dysfunction, promoted by IL-1, also plays a central role in atherogenesis by expression of adhesion molecules and cytokine secretion (3), facilitating leukocyte recruitment and plaque development. The culmination of two decades of preclinical experimental studies in the IL-1 field has led to the ongoing phase 3 clinical trial Cankinumab Anti-inflammatory Thrombosis Outcomes Study, which will test whether blocking IL-1β only will reduce the incidence of thrombotic events in patients after myocardial infarction who remain at high risk because of ongoing inflammation (4).

It has generally been assumed that IL-1β is produced predominantly by immune-derived cells (5). However, we have shown, in ischemic heart disease patients, that atherosclerotic coronary arteries synthesize and express significant IL-1β within the endothelium (6) compared with controls. Experimental studies have also indicated that cultured endothelial cells (ECs)[2] synthesize IL-1β in response to different cytokine stimulations but that the released IL-1β is low and relatively inefficient (7). It is crucial, therefore, to understand the mechanism(s) of release of IL-1β from ECs, especially because IL-1β acts at a distance rather than just locally in the vessel wall.

IL-1β production is a two-step controlled process requiring an "initial" stimulus for transcription/translation of proIL-1β (31 kDa), which, in turn, is cleaved by an inflammasome-activated caspase-1 (8) ("a second hit") into a biologically active isoform (17 kDa) before secretion. The cleavage of proIL-1β is a crucial step, and studies in monocytes show that caspase-1 (a cysteine protease) is a cardinal enzyme in this process (9). There is a spectrum of proposed cellular mechanisms responsible for IL-1 secretion in monocytic cells, including rescue from autophagy and subsequent release, release via microvesicles or multivesicular bodies, and terminal release (via pores), dependent on cell type and stimulus intensity (10).

In vivo studies have postulated that IL-1β can also be released in the absence of caspase-1 (11), suggesting an alternative and still unknown mechanism by which "leaderless" IL-1β is secreted. There are other potential enzymes that cleave proIL-1β into its mature form, including the serine proteases (neutrophil elastase, cathepsin G, and proteinase 3 (12, 13)). It is known

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[2] The abbreviations used are: EC, endothelial cell; NE, neutrophil elastase; HCAEC, human coronary artery endothelial cell; MV, microvesicles; ANOVA, analysis of variance; MVB, multivesicular body; BAF1, bafilomycin A1; NEIII, neutorophil elastase inhibitor III; vWF, von Willebrand Factor.

that, in cell-free systems, these serine proteases cleave purified proIL-1β into a biologically active IL-1β *in vitro* at distinct sites to caspase-1 with production of 18- and 20-kDa isoforms of IL-1β. However, whether and to what extent these proteases can contribute to IL-1β release in cells such as ECs is relatively unknown.

Neutrophil elastase (NE) is a potent serine protease that has wide substrate specificity (14, 15). Experimental studies have potentially focused on the destructive nature of NE, but interesting recent data show that NE can provoke a variety of pro-inflammatory responses, such as IL-8 release from bronchial epithelium and TGF-β production in bronchial smooth muscle cells (14). Moreover, deletion of NE in mice leads to a reduction of serum inflammatory biomarkers such as TNF-α, MCP-1, and IL-1 (16). One study has also demonstrated NE in macrophage-rich human atherosclerotic plaque shoulders (17), and it also appears to be critical in caspase-1-independent IL-1β generation in NE-induced lung (18) and renal injury (19). In this study, we sought to determine whether NE promotes biologically active IL-1β secretion from vascular endothelium. We show that NE stimulation leads to proIL-1β cleavage and increases IL-1β release from coronary artery ECs via a caspase-1-independent, vesicular release-mediated process. Furthermore, we demonstrate that IL-1β is colocalized with NE predominantly in the endothelium in experimental atherosclerosis. This first demonstration and explanation of active IL-1β release from the endothelium potentially provides additional novel strategies for inhibition of IL-1β activity in inflammatory cardiovascular disease.

Experimental Procedures

Human coronary artery endothelial cells (HCAECs) were purchased from PromoCell (Heidelberg, Germany) and cultured in supplemented media according to the recommendations of the manufacturer. The cells, at passages 2–5, were seeded into 6-well plates (2×10^4 cells/well) and grown at 37 °C/5% CO_2 (v/v) until 70% confluent. The first step of stimulation was to up-regulate proIL-1β production by adding cytokines (TNF-α/IL-1α, 10 ng/ml each) for 48 h as described previously (7). Cells were then washed to remove all traces of stimulating cytokines before the media was replaced with serum-free media containing NE (1 μg/ml, equating to 60 IU). To ensure that the stimulating cytokines were removed completely, the final cell wash was tested for the presence of IL-1 via ELISA. No cytokines were detected in these washes (data not shown). In some experiments, cells were preincubated with NEIII (500 μM) (20), caspase-1 inhibitor I (YVAD, 50 μM) (8, 21), or BAF1 (50 nM) (22) for at least 30 min before the addition of NE. At the end of the incubations, supernatants were collected, and the cells were lysed in ice-cold 1% (v/v) Triton X-100 lysis buffer. Both supernatant and cell lysates were stored at −80 °C until the analysis was conducted.

Determination of Cell Viability—Cell viability was evaluated by trypan blue dye exclusion and measurement of lactate dehydrogenase levels in conditioned media. Lactate dehydrogenase detected in cell lysates was used as a positive control for total lactate dehydrogenase. Levels of lactate dehydrogenase were analyzed using a CytoTox 96 non-radioactive cytotoxicity kit (Promega) according to the instructions of the manufacturer.

NE Activity—NE activity was measured spectrophotometrically using a highly specific synthetic substrate (elastase substrate I, MeOSuc-APV-pNA, 100 μM) as described in detail previously (14, 23). Briefly, samples (supernatant and lysate) were added to assay buffer (0.45 Tris base and 2 M NaCl (pH 8.0)) containing elastase substrate I for 6 h. The rate of substrate cleavage was measured using a plate reader (Thermo Scientific) at 410 nm.

ELISA for IL-1β—The concentrations of IL-1β (picograms per milliliter) in the supernatants and lysates were quantified by ELISA Quantikine kits (R&D Systems) according to the recommendations of the manufacturer.

Apoptosis—Apoptosis, detected via caspase-3/7 activity, was analyzed by Caspase-Glo® 3/7 assay (Promega) according to the recommendations of the manufacturer.

Western Blot Analysis for IL-1β Processing and Release—Samples (lysates and concentrated supernatants using 10k Amicon filter devices (Thermo Scientific)) were subjected to Western blotting.

Microvesicle Isolation—MV isolation was conducted as described previously (24). Freshly prepared MVs were used for analysis to avoid false positive effects caused by leakage of contents from MVs damaged by freezing and thawing.

Flow Cytometry Measurement of Extracellular Vesicles—A pellet of extracellular vesicles was resuspended in annexin V-binding buffer and labeled with annexin V PE-Cy7 fluorescence according to the instructions of the manufacturer (eBioscience). Events were acquired using an LSR II flow cytometer (BD Biosciences), and annexin V-positive extracellular vesicles were enumerated using Accu Count beads (Sphero, 0.2–0.9 μm) and analyzed using FlowJo software (TreeStar).

Measurements of Intracellular Calcium Concentration—HCAECs in 96-well plates at a seeding density of 5×10^3 were treated with cytokines or left untreated for 48 h. Fura-4 was then added to the cells according to the instructions of the manufacturer (Invitrogen). After washing off the dye, the cells were incubated with or without NE, and changes in cytosolic Ca^{2+} were recorded using a plate reader (Thermo Scientific) according to the recommendations of the manufacturer. EGTA (6 mM) and ionophore A3784 (10 μM) were used as controls as described previously (25).

Direct Effects of NE on Recombinant IL-1β/ProIL-1β—NE is known to undergo spontaneous autolysis (26) and has a proteolytic activity against many cytokines, such as TNF-α (15). For this reason, we tested the effect of NE with the studied concentration on IL-1β/proIL-1β itself. IL-1β standard (R&D Systems) at a concentration of 125 pg/ml and proIL-1β standard (R&D) at a concentration of 10,000 pg/ml were mixed separately with NE (1 μg/ml) and kept in the incubator for 30 min, 2 h, and 6 h. The samples were stored at −80 °C and tested by ELISA and Western blot analysis.

Detection of Extracellular Vesicular Shedding—HCAECs (2×10^4) were plated in LabTek (Fisher) 8-well chamber slides and subjected to the abovementioned stimulation conditions. Annexin V-Alexa Fluor 488 (Invitrogen) was then added to the cells at 5 μl/well. MV shedding was visualized using image

acquisition software (inverted wide-field fluorescence microscope, Leica AF6000 time-lapse) after the addition of NE in a 5% CO_2/37 °C (v/v) heated chamber. The images were captured after 15 min, 30 min, 2 h, and 6 h and analyzed using Image J software (National Institutes of Health). MVs (0.1–1 μm) were quantified in blinded samples in a random field of cells.

Immunofluorescence—Cells were fixed with 4% w/v paraformaldehyde and permeabilized in 0.3% v/v Triton X-100. Nonspecific binding was blocked for 30 min with 5% v/v goat or rabbit serum in 1% w/v BSA prior to sequential incubation of the cells with the following primary antibodies: anti-goat IL-1β and anti-rabbit LAMP-1 (1:100 dilutions). Alexa Fluor 647- and 488-conjugated secondary antibodies were used in 1% w/v BSA (1:200 dilution). The coverslips were washed with PBS and mounted onto glass slides using media containing DAPI.

The labeling of NE was conducted as described previously (27) using a Microscale protein labeling kit (Molecular Probes) according to the instructions of the manufacturer. 50 μg of NE was used for the reaction. The final concentration of Alexa Fluor 647-NE was 0.1 mg/ml in a volume of 100 μl.

Determination of IL-1β Biological Activity—The bioactivity of the secreted IL-1β by HCAECs following NE stimulation was assessed using an IL-8 luciferase reporter assay sensitive to picomolar concentrations of IL-1β, as described previously (9, 28, 29). Briefly, HeLa cells (5×10^3) were grown to 70% confluence in 96-well plates and transfected with a total of 100 ng of DNA/well, including 60 ng of pIL-8-luc (reporter) and 40 ng of pRL-TK (internal control). After 24 h, the transfection efficiency was assessed, and cells were then stimulated for 6 h with 0.1 nM IL-1β as a control or with harvested supernatants from HCAEC stimulated with NE. IL-1β-neutralizing antibody (1 μg/ml, catalog no. MAB201, R&D Systems) was used in some wells to prove specificity. Cells were then lysed with passive lysis buffer (Promega) and transferred to white well plates to assay for luminescence intensity using LARII and Stop and Glo reagents (Promega). Luciferase activity was calculated by normalizing to the *Renilla* luminescence measured in each well according to the recommendations of the manufacturer (Promega).

Conventional and Immunogold Labeling Electron Microscopy—EC pellets (NE or untreated controls) were processed as described previously (30). Thin sections were immunogold-labeled with anti-goat IL-1β and anti-rabbit LAMP-1 (1:50 dilutions each) primary antibodies for 2 h at room temperature. After washing the grids, the sections were incubated with immunogold-conjugated secondary antibodies (20- and 10-nm gold particles, Agar Scientific) for 2 h. Control experiments were performed using PBS instead of the primary antibodies, and all sections were then post-stained with uranyl acetate and lead citrate.

Mice and Diets—ApoE$^{-/-}$ male mice were bred in-house at the University of Sheffield. Food and water were given *ad libitum* in a controlled environment (temperature, 22–25 °C; humidity, 55% ± 5%; 12-h light cycle). At 8–10 weeks of age, the mice were housed individually and fed a high-fat, Western-type diet containing 21% fat, 0.15% cholesterol, and 0.296% sodium over a 12-week duration. Special Diet Services (Witham, UK) supplied the food. This high-fat diet was used specifically to study the effects of the diet on atherosclerosis, as described previously (31). All animal care and procedures were conducted under Animals (Scientific Procedures) Act 1986 (UK) and approved by The University of Sheffield Ethics Committee. At the end of the study, the mice were euthanized, and proximal aortae were harvested.

Immunohistochemistry—Sections were used for immunohistochemistry as described previously (32).

Statistical Analyses—Results are shown as mean ± S.E. Analyses were performed using GraphPad Prism version 6.04. For multiple-comparison tests, one way analysis of variance (ANOVA) followed by Tukey's test was performed. Statistical significance was achieved when the p value was less than 0.05.

Results

NE Promotes IL-1 Release in HCAECs—To assess the contribution of NE to IL-1β secretion, cytokine-primed ECs were treated with varying concentrations of NE in serum-free media at different time points. As shown in Fig. 1*A*, after 2 h of stimulation, NE at 1 μg/ml caused significant (10×) release of IL-1β from cytokine-primed cells (198 ± 24.85 pg/ml, $p < 0.0001$) compared with primed cells without NE (12.1 ± 4.81 pg/ml). This release decreased significantly with higher concentrations of NE (2 μg/ml) because of a decrease in cell viability (50% ± 10%). Therefore, subsequent experiments used NE at 1 μg/ml to give the highest amount of IL-1β release without a significant increase in cell death (Fig. 1*B*).

After 6 h of stimulation, NE caused a significant increase in IL-1β secretion (Fig. 1*C*) compared with cytokine stimulation alone (272.8 ± 50 pg/ml *versus* 55.5 ± 17.3 pg/ml, $p < 0.001$). No IL-1β was detected in the media of unstimulated cells (Fig. 1*C*).

To specifically confirm that the release was due to NE, the cells were pretreated with the specific neutrophil elastase inhibitor NEIII, resulting in a significant attenuation of IL-1β secretion. To determine the involvement of caspase-1, cytokine-primed ECs were pretreated with YVAD-CHO and then with NE. No significant changes in the secreted levels of IL-1β were seen compared with NE alone (Fig. 1*C*). Therefore, NE-mediated IL-1β release in endothelial cells was independent of caspase-1.

In unstimulated EC lysates, we did not detect any IL-1β. However, there was significant IL-1β production inside cells following treatment with pro-inflammatory cytokines (Fig. 1*D*). The IL-1β levels in the lysates were not significantly different among the groups (Fig. 1*D*). In addition, NE treatment alone did not provoke IL-1β production in the cells (Fig. 1*D*), suggesting no direct effects of NE on IL-1β generation inside the cells. There was also no effect seen on IL-1β production in cell lysates by NEIII or caspase-1 inhibitor (Fig. 1*D*).

To confirm that NE was active over the duration of the study, we measured NE activity using quantitative cleavage of a chromogenic specific substrate and, interestingly, in the lysates harvested from NE-treated cells, this was increased significantly (Fig. 1*E*).

Cell lysis as a mechanism of IL-1β secretion was ruled out by the absence of cytosolic enzyme lactate dehydrogenase levels in

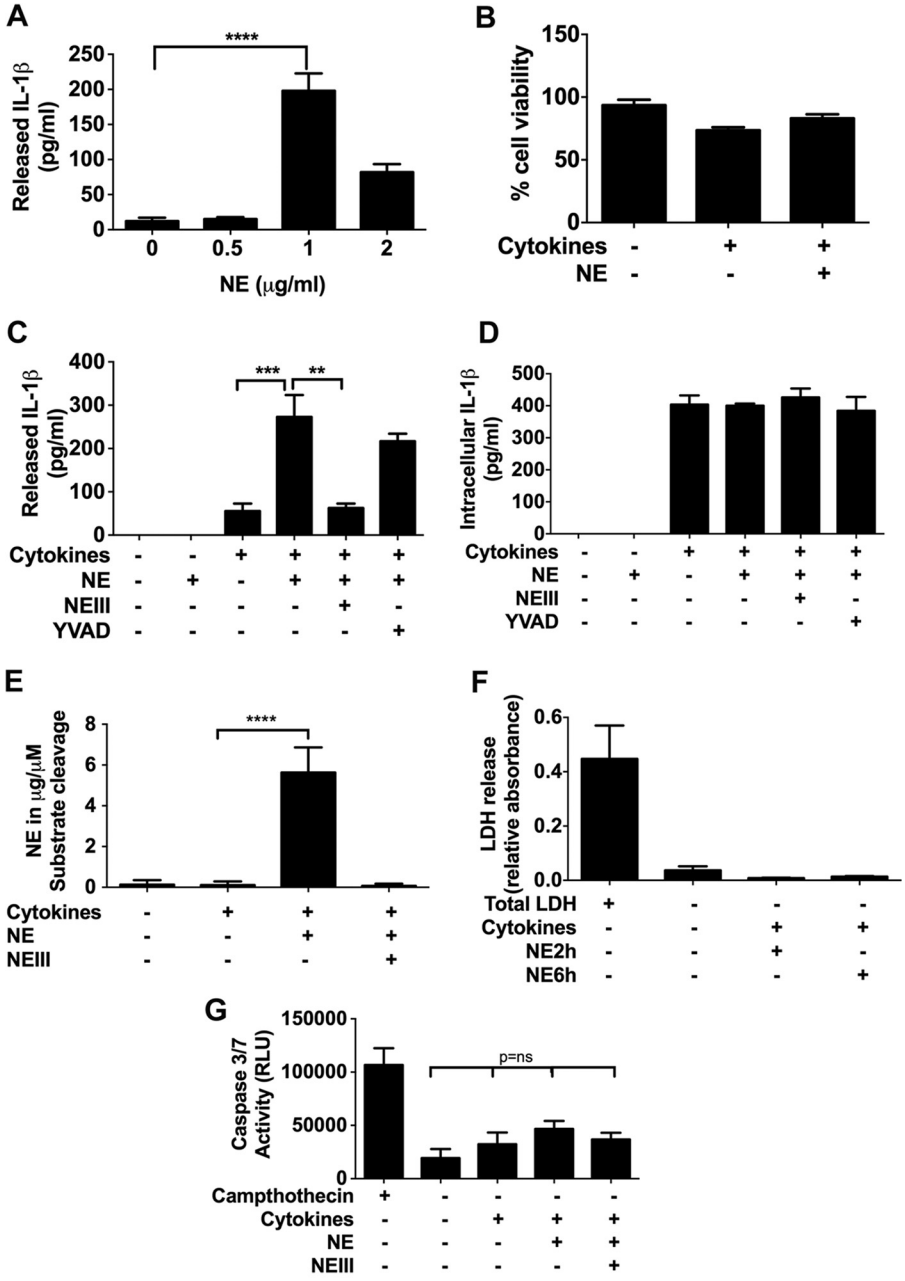

FIGURE 1. **NE enhances IL-1β secretion from HCAECs by a caspase-1-independent mechanism.** *A*, IL-1β is released by HCAECs after 48-h stimulation with cytokines (TNF-α/IL-1α, 10 ng/ml), followed by NE activation (0.5–2 μg/ml) for 2 h as measured by ELISA ($n = 3$). *B*, cell viability (measured by trypan blue) is not reduced significantly following exposure to 1 μg/ml NE ($n = 3$). *C*, HCAECs, incubated for 48 h with or without cytokines and then incubated further for 6 h in serum-free media with or without NE (1 μg/ml) in the presence or absence of inhibitors (NEII, 500 μM; YVAD, 50 μM; $n = 5$), show that IL-1β release is increased by NE independent of caspase-1. *D*, levels of IL-1β in cell lysates are not increased following NE incubation ($n = 5$). E, increased NE activity in EC lysates treated with NE for 6 h compared with unstimulated cells ($n = 3$). *F*, lactate dehydrogenase levels are unchanged following NE treatment, as measured in conditioned media or in cell lysates as a total of lactate dehydrogenase ($n = 3$). *G*, caspase-3/7 activity is unchanged in HCAECs following NE treatment. HCAECs in 96-well plates (2×10^4 cells/well), incubated with or without cytokines (TNF-α/IL-1α, 10 ng/ml each) for 48 h, were subjected to NE (1 μg/ml) in serum-free media for 6 h ($n = 3$). Camptothecin (10 μg/ml) was used to induce apoptosis as a positive control. *RLU*, relative light units. All data are mean ± S.E. and were analyzed by one-way ANOVA with Tukey's multiple comparison test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

cell supernatants. There were no significant changes in lactate dehydrogenase levels following NE stimulation at 2 and 6 h compared with the control (Fig. 1*F*). Because IL-1 release could be a feature of cell apoptosis, we investigated caspase-3/7 activation in ECs under our stimulation conditions, and there was no significant increase in NE-treated cells compared with unstimulated ECs, cytokine-stimulated cells, or cells in which NE effects were attenuated using NEIII (Fig. 1*G*). In addition, we used propidium iodide in conjunction with annexin V to determine whether ECs were viable, apoptotic, or necrotic at any time point in this study. We did not detect any significant increase in propidium iodide/annexin V staining in NE-treated cells compared with untreated controls (data not shown).

In vitro, some proteases, including NE, have been shown to process proIL-1β into mature IL-1β (13). Therefore, because the ELISA does not distinguish between pro- and mature IL-β,

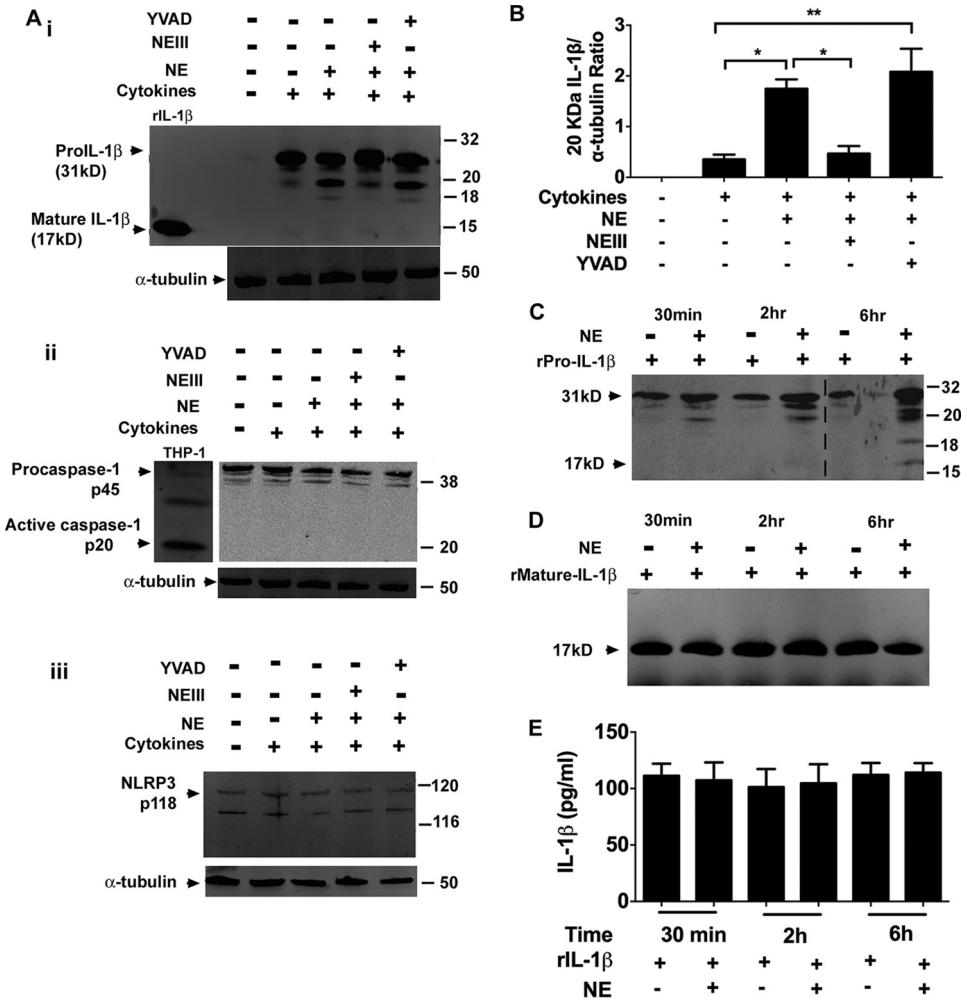

FIGURE 2. **NE selectively cleaves proIL-1β in primed EC lysates without caspase-1/NLRP3 activation.** *A*, Western blot analysis of cell lysates of primed EC with or without NE and assessed for IL-1β (*i*), Caspase-1 (*ii*), and NLRP-3 (*iii*). The blots are representative of three experiments, with α-tubulin levels as a loading control. For IL-1β, recombinant IL-1β (*rIL-1β*, 20 μg, 17 kDa) was loaded as a positive control and represents the commonly detected mature form, whereas proIL-1β (31 kDa) indicates the inactive pro-form. For caspase-1, activated THP-1 cell lysates were used as a positive control for the p20 isoform. *B*, densitometric analysis of 20-kDa IL-1β levels ($n = 3$). *C*, Western blot illustrating the cleavage of recombinant proIL-1β (*rProIL-1β*, 20 μg) by NE. Recombinant ProIL-1β was incubated at 37 °C (5% CO_2, v/v) alone or in the presence of NE (1 μg/ml) for 30 min, 2 h, and 6 h. *D*, Western blot for recombinant mature IL-1β (20 μg) in the presence or absence of NE, showing no cleavage ($n = 3$). *E*, ELISA for recombinant mature IL-1β, showing no difference in levels following NE treatment. Data are mean ± S.E. and were analyzed by one-way ANOVA and Tukey's post-test. *, $p < 0.05$; **, $p < 0.01$.

we determined which IL-1β isoforms were present, and their relative respective levels, in cell lysates by immunoblotting. Unstimulated HCAEC lysates did not contain any detectable IL-1β, but full-length proIL-1β (31 kDa) was seen in cytokine-primed cell lysates (Fig. 2*A*, *i*). However, after the addition of NE, there was cleavage of the 31-kDa pro-form associated with a decrease in the 31-kDa proIL-1β in cell lysates (Fig. 2*A*, *i*) that was NE-specific, as evidenced by inhibition by NEIII but not YVAD. Upon addition of NE, the 20- and 17-kD IL-1β forms were detected (Fig. 2*A*, *i*). Again, this was NE-specific (Fig. 2*B*). Interestingly, NE induced maturation of IL-1β that was not associated with procaspase-1 cleavage or alterations in NLRP-3 levels in lysates, as detected by Western blot analysis (Fig. 2*A*, *ii* and *iii*). Nor did we detect p20 (the product of active caspase-1) in cell media, indicating that cleavage and secretion had not occurred. NE is known for its proteolytic properties. Therefore, to confirm that NE cleaved the pro-form but not the mature form of IL-1, recombinant proIL-1β and recombinant mature IL-1β were incubated with NE in a cell-free system. NE cleaved the pro-form of IL-1 *in vitro* (Fig. 2*C*) but not the mature form (Fig. 2, *D* and *E*), supporting the hypothesis that NE specifically cleaves the pro-form of IL-1β only. Taken together, these data clearly suggest that NE increases cytokine-induced IL-1β secretion in ECs via an inflammasome/caspase-1-independent pathway.

NE Induced Secretion of Extracellular Vesicles Containing Bioactive IL-1 from HCAECs—In immune-derived cells, protected release of IL-1β (microvesicles, multivesicular bodies (MVBs), and exosomes) has been proposed as a mechanism for IL-1β trafficking to the extracellular environment (9, 33, 34). Therefore, we sought to determine whether extravesicular shedding occurred in response to NE and whether this was associated with IL-1β processing in HCAECs.

Because phosphatidylserine exposure has been associated with MV shedding in monocytes (9) and ECs (24), we used annexin V binding (annexin V-Alexa Fluor 488) as a tool to visualize events in live HCAECs. Small particles (0.1–1 μm in diameter) were observed separating from the cells in real time

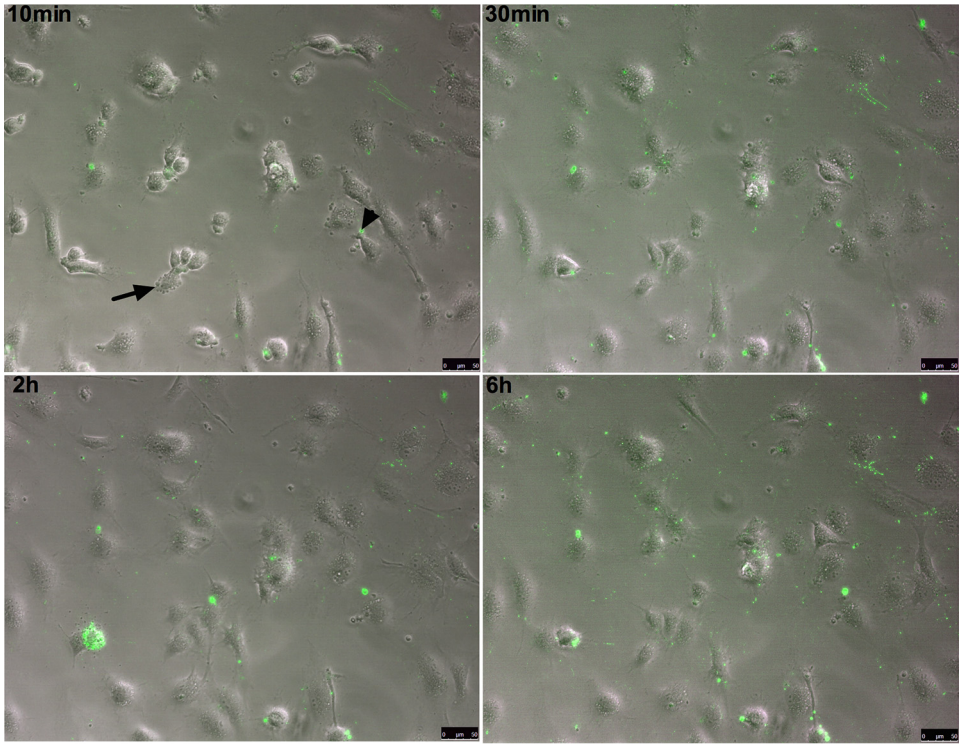

FIGURE 3. **Neutrophil elastase activates microvesicle shedding from endothelial cells in a time-dependent manner.** HCAECs were left untreated or treated with cytokines (IL-1α/TNF-α) for 48 h and then labeled with annexin-V/Alexa Fluor 488. After the addition of 1 μg/ml of NE, cells were visualized in a heated chamber (5% CO_2 v/v) using a confocal microscope to scan MV release. Images captured after 10 min, 30 min, 2 h, and 6 h show an early generation of MVs after 10 min of NE stimulation but more prominent at later time points. The *arrowhead* indicates fluorescent MVs, and the *arrow* indicates earliest blebbing in EC treated with NE. *Scale bars* = 50 μm. The representative images are from three independent experiments and were altered digitally to remove background fluorescence.

using time-lapse imaging over a duration of 6 h (supplemental Movie S1). The first MV generation was at 10 min, with membrane alterations 30 min after NE stimulation, with large numbers of MVs observed at later time points (Fig. 3). The number of MVs was quantified using flow cytometry and gating for annexin V (Fig. 4*A*). Interestingly, there was a significant increase in the number of MVs isolated from ECs following NE treatment (2- to 3-fold) compared with controls. Importantly, NE inhibition effectively attenuated MV formation, and shedding induced by NE and caspase-1 inhibition had no significant effect (Fig. 4*B*).

We next investigated which IL-1β isoforms were inside MVs using immunoblotting. MVs from unstimulated cells contained no IL-1β, and, in MVs from cytokine-primed cells (6 h), prominent proIL-1β (31-kDa) forms were detected (Fig. 4*C*). In MVs isolated from the supernatants of NE-treated cells, cleavage of the 31-kDa IL-1β isoform to ~19–20 kDa was observed from as early as 30 min (Fig. 4*D*), with further cleavage to the 18- and 15-kDa isoforms after 6 h (Fig. 4*C*). Treatment of cells with NEIII abolished any cleavage of proIL-1β in these MVs, confirming that these bands are the result of direct NE activity. Cleavage of proIL-1β continued even in the presence of YVAD, with more prominent isoforms detected (Fig. 4*C*). MVs were assessed for caspase-1 and NRLP3 content, and, interestingly, active caspase-1 p20 and NLRP3 were not detected in MVs isolated from cells treated with NE or NE and YVAD together (data not shown), indicating that intravesicle cleavage of proIL-1β is independent of caspase-1 activation. These findings suggest that either NE cleaves the released proIL-1β inside MVs or that NE-treated cells continually generate more MVs containing IL-1β as a route of secretion.

We next asked whether the processed IL-1β released into cell supernatants in vesicles as a result of NE activation was bioactive. We collected total supernatants (containing MVs) from NE-treated or untreated cells for 6 h, applied them to HeLa cells expressing an IL-1RI-responsive reporter, and measured IL-8 activity. We compared the reporter assay output (IL-8) with media obtained from unstimulated or cytokine-primed EC ± NE with a positive control (0.1 nM recombinant IL-1β) or with an IL-1β-neutralizing antibody. As shown in Fig. 4*E*, supernatants isolated from NE-activated ECs increased significantly IL-8 activity compared with unstimulated and cytokine-primed cells, and this was abrogated completely by the neutralizing antibody. To confirm that the bioactivity was due to released IL-1β and not a result of direct NE effects on HeLa cells, NE (1 μg/ml) was added to HeLa cells (as a spike), and this showed no significant IL-8 activation. These data indicate that the IL-1β in the MVs is bioactive. MVs containing IL-1β were confirmed using immunogold transmission electron microscopy, and we only detected 0.2-μm-diameter MVs containing immunogold-labeled IL-1β in NE-treated cells (Fig. 4*F*).

Mechanisms of IL-1β Release in Endothelial HCAECs—To study early MV shedding by NE, intracellular calcium changes were assessed. MV release has been linked with transient changes in intracellular calcium ($[Ca^{2+}]_i$) in specialized secretory cells (35) and IL-1β secretion in macrophages (33). Using a

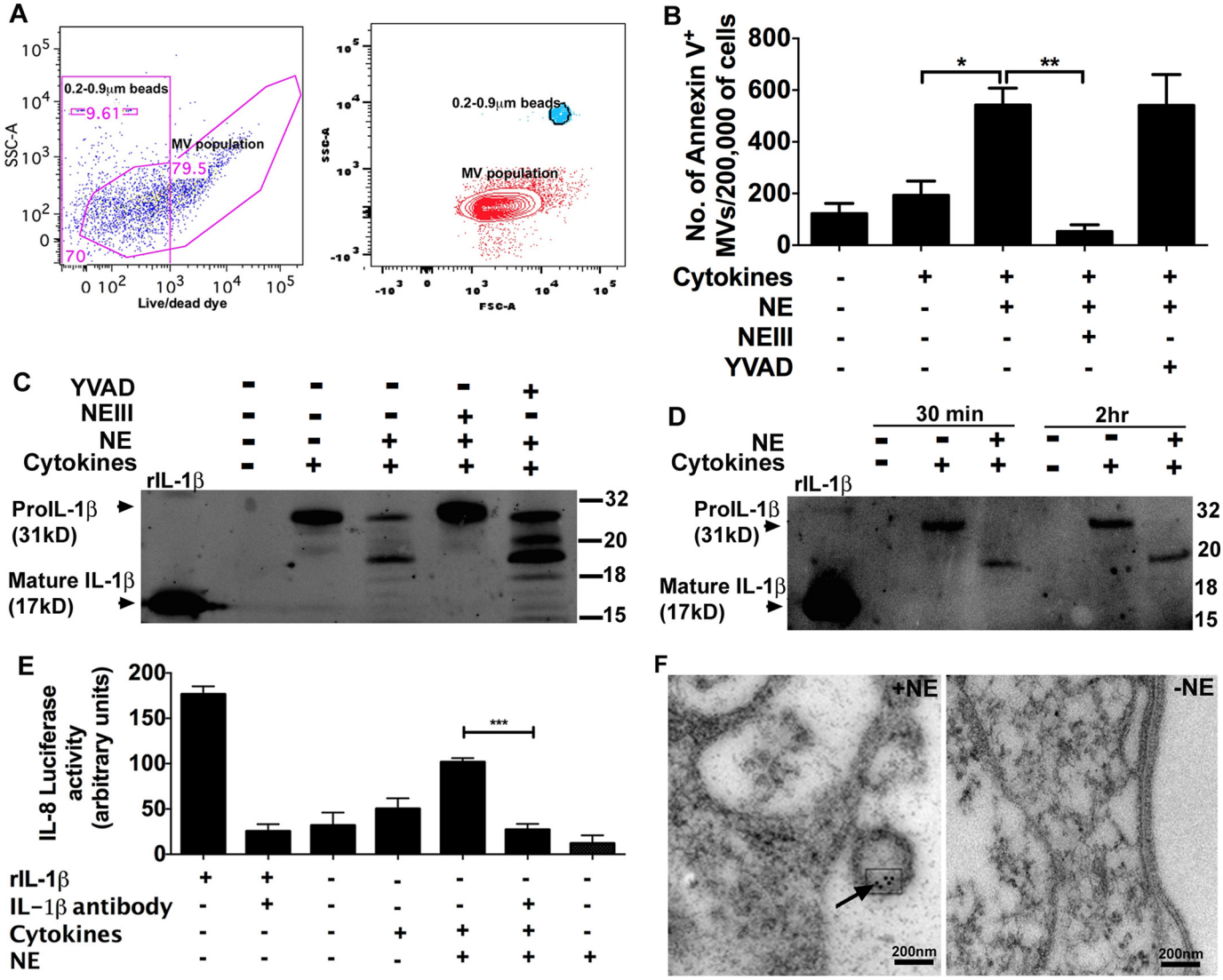

FIGURE 4. **NE induces secretion of extracellular vesicles containing bioactive IL-1 from HCAECs.** *A*, flow cytometric characterization of MVs released in response to NE. MVs were isolated and stained with annexin V/PE-Cy7 as described under "Experimental Procedures." Analysis of MVs (*red*) using Megamex beads (*blue*) shows that they are within the 0.2- 0.9-μm size limit. *B*, a significant increase in MVs stained with annexin V is seen in NE-treated cells compared with untreated controls. Analysis was performed by FlowJo software ($n = 3$). *FCS*, forward scatter. Data are mean ± S.E., one-way ANOVA followed by Tukey's post- test. *, $p < 0.05$; **, $p < 0.01$. *C* and *D*, detection of IL-1β in isolated MVs by immunoblotting. Equal amounts of protein (20 μg) were loaded in each lane, with recombinant IL-1β (*rIL-1*β, 20 μg) used as a positive control (17 kDa). Data are representative of four experiments. *E*, luciferase assay for the measurement of IL-1β bioactivity in HeLa cells exposed to freshly harvested conditioned media (total supernatants from cytokine-primed cells (TNF-α/IL-1α, 10 ng/ml each with or without NE, 1 μg/ml) or recombinant IL-1β (0.1 nM) for 6 h with or without anti-IL-1β (1 μg/ml). Specificity for IL-1β is shown by a reduction of IL-8 luciferase detection following incubation with IL-1β-neutralizing antibody. Data are expressed as mean ± S.E. ($n = 3$) and were analyzed by one-way ANOVA followed by Tukey's test. ****, $p < 0.0001$. *F*, immunoelectron microscopic analysis of IL-1β in ECs ± NE treatment. Anti-IL-1β-conjugated immunogold (20-nm gold particles, *arrow*) was used to confirm the presence of IL-1β in the MVs (0.2 μm) released from the plasma membrane of ECs.

Ca^{2+}-sensitive fluorometric dye, we assessed the role of $[Ca^{2+}]_i$ in MV formation and release in response to NE and performed experiments in the presence or absence of exogenous Ca^{2+}. In this experiment, $[Ca^{2+}]_i$ is released from intracellular stores during an initial stimulation/treatment in Ca^{2+}-free media, and application of $CaCl_2$ during the second phase of the protocol allows Ca^{2+} influx inside ECs. In Ca^{2+}-free media, there was a small non-significant increase in cytosolic Ca^{2+} levels in NE-treated cells compared with untreated cells after NE stimulation (Fig. 5*A*). However, $[Ca^{2+}]_i$ was increased significantly in NE-stimulated cells after the addition of $CaCl_2$ compared with unstimulated and cytokine-primed cells (Fig. 5*B*). This finding suggests that NE treatment of ECs increases free $[Ca^{2+}]_i$ by promoting Ca^{2+} influx into ECs and that this calcium influx is associated with MV release.

We detected small MVs (~200 nm) in response to NE (assessed by flow and electron microscopy), characteristic of exosomes secreted from MVBs, and this led us to study the compartmentalization of IL-1β in ECs (30). HCAECs ± NE were stained using an immunofluorescence technique for LAMP-1 (a late endolysosomal marker) and IL-1β. Confocal images of cytokine-primed ECs stained for IL-1β and costained for LAMP-1 suggested a wide distribution of IL-1β throughout the cytoplasm and no colocalization with LAMP-1 (Fig. 5*C*). Not surprisingly, no signals were detected for IL-1β in unstimulated ECs (data not shown). However, in ECs incubated with NE

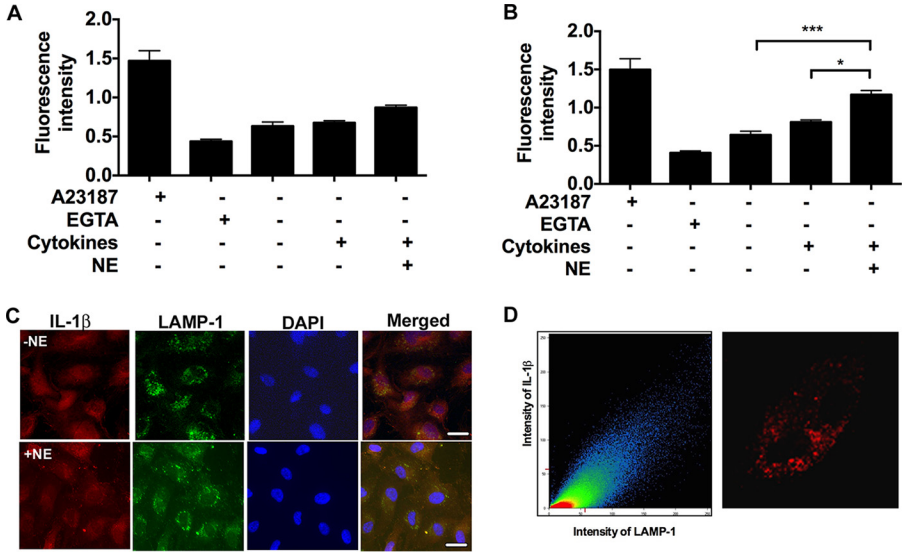

FIGURE 5. **Mechanisms contributing to IL-1β release in endothelial HCAEC.** *A* and *B*, HCAECs were assayed for changes in cytosolic free Ca^{2+} in response to the indicated conditions. *A*, no significant change in cytosolic Ca^{2+} levels are seen in Ca^{2+}-free media. *B*, the fluorescence intensity of intracellular calcium changes after 5 min of NE stimulation in the presence of $CaCl_2$. Data are mean ± S.E. ($n = 6$) and were analyzed by one-way ANOVA and Tukey's post-test. *, $p < 0.05$; ***, $p < 0.001$. *C*, IL-1β colocalizes with LAMP-1 after NE stimulation. Cells were primed with cytokines (TNF-α/IL-1α, 10 ng/ml each), followed by incubation ± NE (1 μg/ml) in serum-free media over 2 h before immunostaining for IL-1β (*red*) and LAMP-1 (*green*). *Scale bars* = 10 μm. *D*, histogram showing a high correlation between IL-1β and LAMP-1 in ECs after NE activation.

for increasing times (30 min, 1 h, and 2 h), IL-1β was colocalized with LAMP-1 (Fig. 5*D*). Indeed, following a 2-h stimulation of EC with NE, the majority of IL-1β was detected in MVBs (Fig. 5*D*).

To confirm that the site of IL-1β processing and secretion was indeed mediated by an endolysosomal mechanism, we evaluated the effect of bafilomycin A1 (BAF1), a lysosomal V/AT-Pase inhibitor that has been shown to prevent endolysosomal formation (36). Treatment of ECs with BAF1 (50 nM) before the addition of NE largely decreased IL-1 levels in the supernatants after 6 h, and that was associated with a reduction in pro-IL-1 cleavage in the lysates (Fig. 6, *A* and *B*). We subsequently performed transmission electron microscopy on cytokine-primed HCAECs ± NE and observed >200 nm structures in the cytosol with classical morphological features of MVBs (37). These were detected in close proximity to the plasma membrane in NE-activated ECs but not in unstimulated ECs (Fig. 6*C*, *i*). Interestingly, inside the cells, the majority of IL-1 was detected within the MVBs (Fig. 6*C*, *ii*).

NE Is Detected in ECs and Is Colocalized with IL-1β in the Endothelium of Mature Atherosclerotic Plaques—To follow the fate of NE in activated ECs, we used Alexa-Fluor 647-labeled NE and performed immunofluorescence staining. After permeabilization, we also labeled the internal endolysosomes with LAMP-1. Surprisingly, NE was detected inside cells. The enzyme colocalized with LAMP-1 (Fig. 7*A*).

Finally, we asked whether NE could be detected in atherosclerotic plaques in mice to ascertain whether NE could contribute to local IL-1β generation. Only in well developed atheromatous lesions of $ApoE^{-/-}$ mice fed a high-fat diet for 12 weeks was IL-1β detected, predominantly in endothelial cells. Interestingly, in these lesions, NE also appeared to be expressed in the luminal endothelium (Fig. 7*B*) and colocalized with IL-1β-positive stained ECs (Fig. 7*C*).

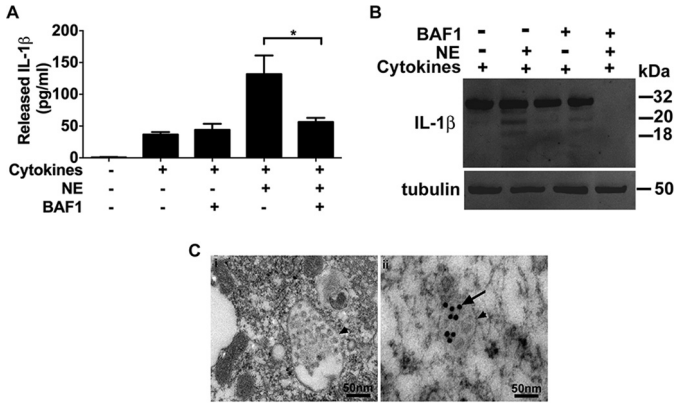

FIGURE 6. **NE induces IL-1β release by an endolysosome-dependent mechanism.** *A*, ELISA measuring IL-1β release in conditioned media of HCAECs primed with cytokines (TNF-α/IL-1α, 10 ng/ml) with or without NE (1 μg/ml) or BAF1 (50 nM) after 6 h. Data are mean ± S.E. ($n = 3$) and were analyzed by one-way ANOVA followed by Tukey's post-test. *, $p < 0.05$. *B*, Western blot analysis of lysates harvested from primed HCAECs activated with NE with or without BAF1 (50 nM) for 6 h. 20 μg of protein was loaded per lane, with α-tubulin used as a loading control. The blot is representative of three independent experiments. *C*, MVB characterization. *i*, EM analysis showing a full appearance of MVBs in NE-treated cells in close proximity of the plasma membrane. *ii*, immunolabeling with anti-IL-1β (20-nm gold particles, *arrow*), showing IL-1β within MVBs (*arrowhead*).

Discussion

Here we describe, for the first time, how coronary artery ECs release IL-1β, which has been a "holy grail" of endothelial biology for many years. We report that a considerable amount of IL-1β is released from ECs in response to NE via a caspase-1-independent vesicular pathway. This is supported by the following lines of evidence: bioactive IL-1β is secreted only in MVs following NE treatment, IL-1β secretion is unaffected by YVAD and occurs without caspase-1/NLRP3 activation, and MVB are prevalent in primed NE-treated ECs and contain IL-1β protein. We propose that "protected release" (10) (*i.e.* contained within

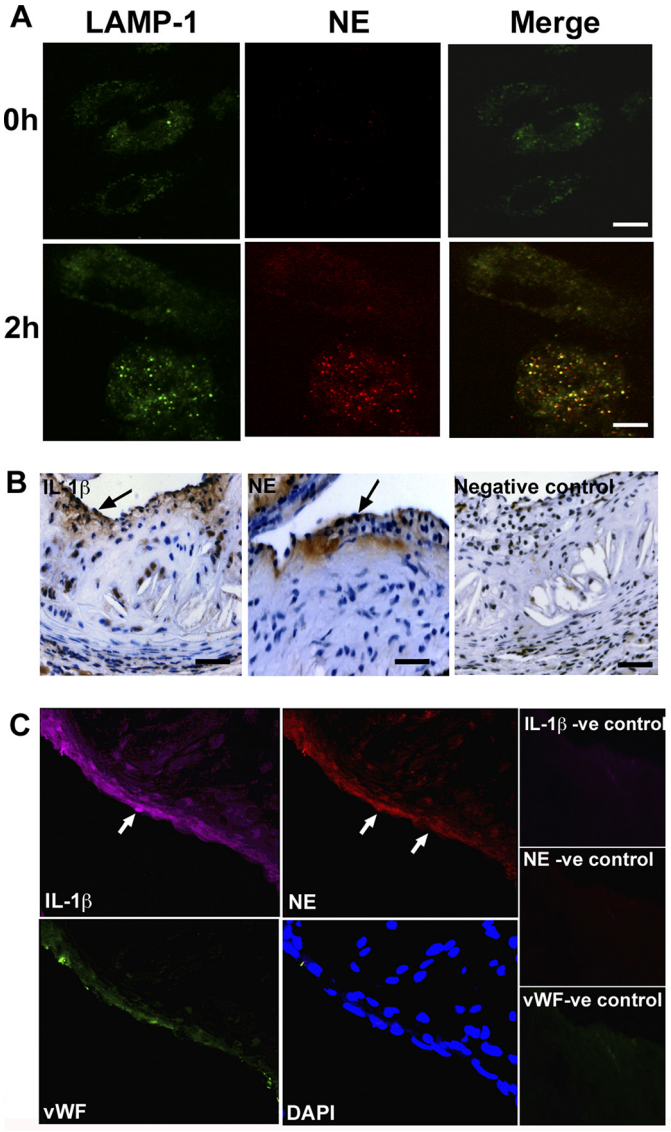

FIGURE 7. **NE is detected in ECs and is colocalized with IL-1β in the endothelium of mature atherosclerotic plaques.** *A*, confocal images showing LAMP-1 and NE in primed ECs after NE treatment. HCAECs were incubated with or without Alexa Fluor 647-labeled (1 μg/ml) NE for 2 h in serum-free media before washing in PBS, and colocalization was performed using an antibody against LAMP-1. Confocal images were analyzed using Zeiss image and ImageJ software. *Scale bars* = 10 μm. *B*, immunohistochemical detection of NE and IL-1β in the luminal endothelium of mouse atherosclerotic plaques. Paraffin-embedded aortic sinuses from ApoE$^{-/-}$ mice fed a high-fat diet for 12 weeks were stained with primary antibodies as indicated. The specificity of staining was confirmed by no primary negative control. *Scale bars* = 200 μm. *C*, colocalization of IL-1-β, NE, and von Willebrand Factor (vWF) in aortic atherosclerosis. NE positivity was detected predominantly in the endothelium (*top right panel*, *arrows*). IL-1β positive endothelium (top left panel) was also detected. The *bottom panels* show vWF-stained endothelium, and DAPI was used for the nuclei. The specificity of staining was confirmed by no primary negative control. Images are representative of histology data obtained from a total of six animals.

membrane-bound vesicles) of IL-1β is a prevalent mechanism in HCAECs.

The endothelium is fundamental in atherosclerotic plaque development not only during early lesion development but also later by controlling plaque instability (38). In atherosclerosis, cross-talk between circulating cells such as monocytes and neutrophils and the endothelium can cause ECs to liberate soluble agents, perpetuating the cycle of inflammation. Several lines of evidence suggest that IL-1 is an apical cytokine in this process (39), but its mechanism of release from ECs is largely unknown. Furthermore, the biological pattern of the cross-talk has not been completely defined. We hypothesized that NE induces IL-1β secretion from the vascular endothelium.

Because caspase-1 has been identified as the main proteolytic enzyme to play a role in proIL-1β cleavage and secretion in monocytes and macrophages (40), we used a specific caspase-1 inhibitor (YVAD-CHO) as a potential means of attenuating IL-1β release. Our data show that caspase-1 appears to be non-essential in EC in this setting for IL-1β cleavage and release by NE. This is at odds with other *in vitro* studies in monocytes (21) but in agreement with more recent data from other cell types (41) and *in vivo* models (42, 43). Our findings are also supported by the findings of Guma *et al.* (11), who describe the presence of IL-1β in the synovial fluid of caspase-1$^{-/-}$ mice. Moreover, our data may explain why caspase-1 suppression did not show promise in vascular healing or atherosclerosis progression (32).

It has been shown previously that IL-1 lacks the signal peptide for directing it to the classical endoplasmic reticulum-Golgi secretory pathway (30). Therefore, IL-1β release has been proposed to occur by distinct mechanisms, including MV shedding and endolysosomal regulation (10). Strikingly, in HCAECs, NE-induced MV shedding occurred independently of caspase-1 activity, which is in contrast to previous investigations using immune cells which reported caspase-1 dependent MV shedding. We suggest that, at least in HCAECs, NE is able to directly cleave the IL-1β precursor, which is associated with protected release in membrane-bound vesicles. In MVs, the prominent forms of IL-1β released (also present inside cells) were 20, 18, and 15 kDa. These isoforms have been detected previously *in vitro* (13), and, although proposed as 5- 10-fold less bioactive than the 17-kDa isoform, they are active enough for IL-1 to bind to its receptors and initiate signaling. Our study is the first to show, in intact cells, that NE is capable of cleaving proIL-1β at multiple sites and that these products are bioactive.

A common biogenesis has linked Ca^{2+}-regulated MV shedding and IL-1 secretion with an increase in intracellular calcium levels (33). We are in agreement with this and demonstrated that NE transiently increased $[Ca^{2+}]_i$ to a maximum after addition of exogenous Ca^{2+}, suggesting that NE mobilized $[Ca^{2+}]_i$ mainly by influx of extracellular Ca^{2+}. The secretory pathway identified here has been used to describe the secretion of other non-classical proteins, such as EGF (44). Although a few studies have begun to investigate NE-mediated IL-1β secretion in renal and lung disease, ours is the first to propose a direct effect of NE on IL-1β release in cardiovascular inflammation and to link this to a MV release mechanism.

Recent immunofluorescence staining data in murine macrophages have shown that IL-1β does not colocalize with LAMP-1 in macrophages (45). This finding is consistent with our data in ECs prior to NE treatment (cytokine-primed cells) where IL-1β appears to be distributed diffusely within the cytoplasm. Significantly, however, 2 h after NE treatment, colocalization increased, strongly suggesting that IL-1β compartmentalization may be induced acutely by NE and predestined for maturation by regulated transport. In ECs primed with cyto-

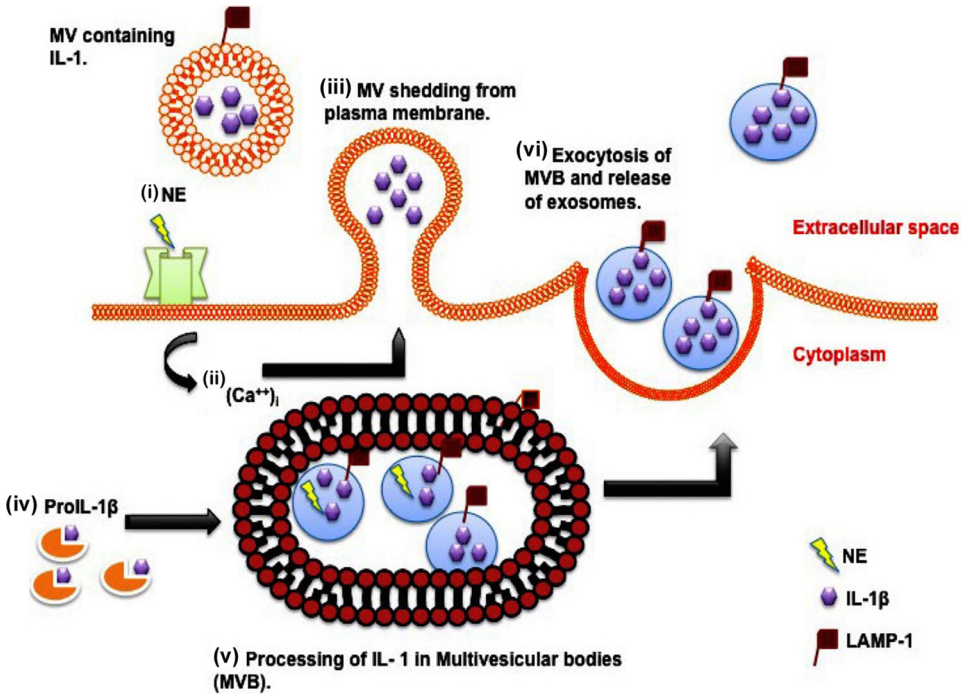

FIGURE 8. **Schematic of the proposed mechanism of IL-1β secretion from ECs by NE.** NE is released by circulating cells at the site of atheroma and transported by endocytosis inside the diseased endothelium (primed by inflammation) (*i*). An increase in calcium because of NE effects leads to remodeling of the cell membrane and vesiculation (*ii*), which, in turn, facilitates the shedding of MVs containing mature IL-1β (*iii*). ProIL-1β is up-regulated in the inflamed endothelium (*iv*). NE enters MVBs and cleaves the proIL-1β contained within (*v*). MVBs also fuse to the plasma membrane and release exosomes containing IL-1 (*vi*).

kines and treated with NE, we observed that MVBs with cytosolic IL-1β accumulated within cell invaginations, similar to that shown in Ref. 46. This is consistent with work from Rubartelli *et al.* (47) in monocytes. Because MVBs were not detected in cells that did not produce IL-1β (33), this strongly suggests that MVBs are a part of the secretory pathway of IL-1β.

The colocalization of IL-1β and LAMP-1 suggests that NE may trigger a signaling pathway that allows the IL-1β processing to occur in secretory endolysosomes (MVBs). However, the possibility of NE internalization within ECs, via endocytosis, cannot be ruled out, particularly because we detect enhanced NE activity in NE-treated ECs lysates compared with untreated and cytokine-primed cells. In agreement with others who have shown that NE is internalized by macrophages (48) and tumor cells (27) by active transport, we also detected NE inside ECs using confocal imaging. Therefore, it is possible that, after internalization of NE by ECs, NE enters MVBs, where it cleaves proIL-1β.

It is possible that NE also causes the release of IL-1β because of a toxicity effect, with NE causing cell damage and leakage of cellular contents. However, at the NE concentration used, this is unlikely to be the case because cell viability was not affected significantly, nor was apoptosis proven. Indeed, IL-1β has been shown to increase caspase-3/7 in cells without inducing apoptosis (49), which is in agreement with our data. NE increases IL-1β levels, which, in turn, affects caspase-3/7 but does not affect apoptosis. We use recombinant NE throughout this study, raising the question of what the natural source of NE would be and whether levels of NE would be enough to activate ECs. Previous work by our group has shown that IL-β is released from activated cocultures of ECs and monocytes at greater levels than from monocytes or ECs alone and that an unidentified monocyte-derived mediator contributed significantly to this response (50). Taken together with the findings reported here, we postulate that NE is the mediator released from monocytes and that it would be the natural source of NE *in vivo*.

Given the continued prominence and topicality of IL-1 in the generation and progression of atherosclerosis (51), we studied the expression of NE *in vivo* in a recognized atherosclerosis preparation: aortic root plaques taken from ApoE$^{-/-}$ mice fed a high-fat diet for 12 weeks. Although previous work has detected NE in coronary arteries (6), aortic aneurysms (52), and carotid plaques (17), our study is the first to investigate NE distribution in experimental atherosclerosis. Significantly, NE was mainly detected in the endothelium of plaques and was detected alongside IL-1β. The antibody used for these studies recognizes both the proIL-1β and mature forms, and we show a clear cellular colocalization with NE in support of our data showing that NE activates and promotes the secretion of IL-1β. This colocalization of NE and IL-1β suggests that the IL-1β observed is likely to be active. We propose that circulating NE is assimilated into developing plaques from degranulating immune cells during their passage through the main vasculature or via the vasa vasorum, where these exist. This suggestion remains to be clarified, and a causal connection between IL-1β and NE needs to be confirmed in a future study utilizing NE$^{-/-}$ mice.

In conclusion, we significantly add to and cement the emerging role of NE in IL-1-induced inflammation. Furthermore, we suggest a novel mechanism for NE-mediated IL-1 secretion by ECs; namely, proIL-1 processing in the secretory endolyso-

somes and packaging of mature bioactive IL-1 within MVs for release into the extracellular environment as part of a single continuum mechanism (Fig. 8), which is similar to that proposed previously for other cell types (10). We detect NE and IL-1β *in vivo* in the setting of atherosclerosis within the endothelium in atheromatous plaques. The pathophysiological relevance of the detection of vesicular IL-1, particularly derived from the endothelium, gives ECs the potential to exert a regulatory influence upon atherogenesis and, therefore, to become a possible therapeutic target by modulating IL-1 secretion in the local environment. Our findings have a wide application for a better understanding of the role of other important proteases with prominent non-proteolytic and, possibly, signaling roles, such as azurocidin, proteinase 3, and Cathepsin G, and provide other avenues for therapeutic targets to limit the influence of interleukin-1.

Author Contributions—M. A. performed all experiments, analyzed the results, and contributed to writing the paper. H. W., M. D., A. B., and V. R. helped design, conduct, or analyze experiments involving microvesicles. J. C. and S. F. conceived and coordinated the study and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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